Comparison of Responses in the Murine Local Lymph Node Assay (LLNA) Between CBA and BALB/c Mouse Strains

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Abstract

While CBA is currently the recommended strain for the LLNA, the assay was originally developed using BALB/c mice. Since the introduction of the LLNA, several groups in the U.S. have published LLNA studies using BALB/c mice, including the National Toxicology Program, the National Institute for Occupational Safety and Health, and the Dow Chemical Corporation. This has resulted in reference databases for the LLNA that include studies conducted with both CBA and BALB/c mice. However, there is little published literature that directly compares the performance of the LLNA in studies done on the same substances in the two mouse strains. The study reported here is a retrospective evaluation of the results of LLNA studies using CBA mice compared to results using BALB/c mice. NICEATM evaluated 108 independent studies representing 16 substances in four vehicles in which 86 studies used CBA mice and 22 used BALB/c mice. Fourteen of these substances had guinea pig reference data and 13 had human reference data. LLNA outcomes using BALB/c are in agreement with LLNA outcomes obtained with CBA for 81% (13/16) of the test substances. LLNA outcomes with CBA agree with guinea pig outcomes for 86% (12/14) of the test substances and with human outcomes for 85% (11/13) of the test substances. LLNA outcomes with BALB/c agree with guinea pig outcomes for 72% (10/14) of the test substances and with human outcomes for 69% (9/13) of the test substances. A correlation analysis of log transformed EC3 values calculated using LLNA data from each of the two strains indicates that the results from the two strains are correlated ($r = 0.79$, $p \leq 0.0005$). Where there were different outcomes (n=3) between the two mouse strains, the CBA studies were positive while the BALB/c studies were negative. Because the CBA study results were concordant with the human and GP outcomes, these results suggest that further characterization of strain and substrain differences is needed. ILS staff was supported by NIEHS contract N01-ES-35504.

Introduction

CBA mouse

In 1999, the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) recommended to U.S. Federal agencies that the LLNA is a valid substitute for currently accepted guinea pig test methods to assess the allergic contact dermatitis potential of many types of substances (Haneke et al. 2001). The LLNA provides several advantages compared to guinea pig methods, including elimination of potential pain and distress, use of fewer animals, less time required to perform, and availability of dose-response information (Dean et al. 2001; Sailstad et al. 2001). The recommendation was based on a comprehensive evaluation that included an independent scientific peer review panel assessment of the LLNA validation status (ICCVAM 1999).

The LLNA was subsequently incorporated into national and international test guidelines for the assessment of skin sensitization (OECD 2002; ISO 2002; EPA 2003) and is now commonly used worldwide. The recently updated ICCVAM-recommended LLNA protocol states that mouse strains other than CBA may be used in the LLNA if it is sufficiently demonstrated that these animals perform as well as CBA mice in the LLNA (ICCVAM 2009).

Although CBA mice are currently recommended as the preferred mouse strain in national and international LLNA test guidelines, the LLNA was originally developed using BALB/c mice (Kimber et al. 1986). Kimber and Weisenberger (1989) observed that *in vitro* proliferation of lymph node cells in response to exposure to 2,4-dinitrochlorobenzene was stronger in CBA/Ca mice than in BALB/c, and chose to focus on using CBA/Ca mice in further development efforts for the LLNA.

BALB/c mouse

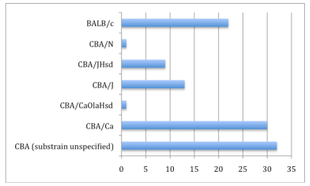
Woolhiser and co-workers assessed LLNA responses in various mouse strains including CBA and BALB/c. They found essentially equal levels of lymph node proliferation (as measured by incorporation of ^{3}H-thymidine into the draining auricular lymph nodes) in both strains following exposure to the sensitizers α-hexylcinnamaldehyde (HCA), 2,4-dinitrofluorobenzene (DNFB) and toluene diisocyanate (Woolhiser et al. 2000).

Other U.S. groups have published LLNA studies using BALB/c mice, including the National Institute for Occupational Safety and Health, the Dow Chemical Corporation, and the National Toxicology Program (Anderson et al. 2009; Bovenhof et al. 2009; NTP 2005).

Database Description

- The database contains results from a total of 108 independent LLNA studies.
  - 15 different test substances
  - 86 CBA studies
  - 22 BALB/c studies
- A frequency distribution of each substrain (to the extent this information is available) is shown in **Figure 1.**
- Suppliers of mice are shown in **Table 1.**
- Four different vehicles were used among the 108 studies:
  - Acetone-olive oil (AOO, 80 studies)
  - Dimethyl sulfoxide (DMSO, 17 studies)
  - Acetone (ACE, 7 studies)
  - Dimethylformamide (DMF, 4 studies)
- Only one nonsensitizer (as classified by results in guinea pigs and humans), methyl salicylate, was included.
- EC3 values (as determined from CBA LLNA data) ranged from 0.0018% (oxazolone in AOO) to 18.2% (eugenol in ACE).

Figure 1: Substrain Frequency Distribution



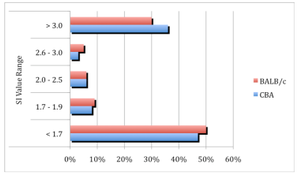
Methodology

- Data included in this study were extracted from published reports or submitted to NICEATM in response to a *Federal Register* (*FR*) notice (72 FR 27815, available at http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/FR/FR_E7_9544.pdf).
- With some exceptions, the data included in the evaluation were generated using the LLNA protocol outlined in the Organisation for Economic Co-operation and Development (OECD) Test Guideline (TG) 429 (OECD 2002).
- Since many of the BALB/c studies were done prior to formal adoption of OECD TG 429, exceptions included:
  - Studies in which lymph nodes were harvested on days 3, 4, 5, or 6 after study initiation.
  - Studies that used 2 or 3 mice per treatment group.
- Studies that included other modifications (e.g., pretreatment of mice with sodium lauryl sulfate before application of the test substance) were excluded.
- An LLNA result was deemed positive if a stimulation index (SI) ≥ 3.0 occurred at any test concentration.
- Since this was a retrospective study, there were substances with multiple studies using the same strain. For each such substance, LLNA outcome was based on the most prevalent study result (positive vs. negative), or considered positive if an equal number of positive and negative studies were found.
- EC3 values (the estimated concentration of a test substance associated with an SI value of 3) were calculated according to Ryan et al. (2007).
  - For some positive studies (i.e., SI ≥ 3.0), an EC3 value could not be calculated due to inadequate dose response (i.e., very low slope or nonmonotonic dose-response).
  - However, these results were still used for the purpose of calculating agreement between strains.

Comparison of Responses in the LLNA from CBA and BALB/c Databases

- Initially, results from LLNA studies using CBA mice (75 substances, 83 LLNA studies) were compared to results from LLNA studies using BALB/c mice (39 substances, 41 LLNA studies) (ICCVAM 2009).
- The percentage of positive LLNA studies (i.e., SI ≥ 3.0) using either CBA (59% [49/83]) or BALB/c (63% [26/41]) mice was similar.
  - **Figure 2** shows the frequency distribution of LLNA responses from 277 test substance doses that fall into the indicated ranges of SI values.
  - However, this does not include a comparison of results from the same substances tested in the same vehicles.
- The study described in this poster was done to compare results of substances tested in the same vehicle in both CBA and BALB/c strains.

Figure 2: Comparison of LLNA Response from CBA and BALB/c Databases (277 Test Substance Doses)



Abbreviations: LLNA = murine local lymph node assay; SI = stimulation index. Data from ICCVAM (2009).

Table 1: Suppliers of Mice Used in LLNA Studies

Mouse Strain	Supplier	No. Studies
CBA (substrain unspecified)	Taconic Laboratories, Germantown, NY	1
	Jackson Laboratories, Bar Harbor, ME	2
	Unspecified	29
CBA/Ca	B&K Universal AB, Sollentuna, Sweden	2
	Harlan Sprague Dawley, Inc., Frederick, MD	3
	Barriered Animal Breeding Unit, Adderly Park, UK	1
	Unspecified	6
	Harlan Olac, Bicester, Oxfordshire, UK	18
CBA/CaOlaHsd	Charles River Laboratories, Inc., Kingston, NY	1
CBA/J	Charles River, Germany	2
	Unspecified	2
CBA/JHsd	Harlan Sprague Dawley Inc, Indianapolis, IN	9
	Harlan Sprague Dawley, Inc., Frederick, MD	8
	Unspecified	1
	Japan SLC Inc, Shizuoka, Japan	1
CBA/N	Jackson Laboratories, Bar Harbor, ME	1
BALB/c (substrains unspecified)	Jackson Laboratories, Bar Harbor, ME	1
	Charles River Japan Laboratories, Atugi, Kanagawa, Japan	2
	Charles River, Germany	3
	Taconic Laboratories, Rockville, MD	1
	Taconic Laboratories, Germantown, NY	2
	Japan SLC Inc, Shizuoka, Japan	4
	Harlan Olac, Bicester, Oxfordshire, UK	4
	Charles River Laboratories, Inc., Kingston, NY	1
	Charles River, Raleigh, NC	1
	Charles River Laboratories - location unspecified	2

Table 2: Summary of LLNA Responses from CBA and BALB/c Mouse Strains

Test Substance	Vehicle	No. of Studies							Avg. EC3 (%)	
		All Strains	CBA			BALB/c				
		Total	Total	Pos	Neg	Total	Pos	Neg	CBA	BALB/c
3-Amino-5-mercapto-1,2,4-triazole	DMSO	2	1	1	0	1	1	0	11.6	5.2
Benzocaine	AOO	5	4	1	3	1	0	1	NC	NC
Cobalt chloride	DMSO	3	2	2	0	1	0	1	0.6	NC
2,4-DNCB	AOO	14	10	10	0	4	4	0	0.052	0.116
2,4-DNFB	AOO	3	1	1	0	2	2	0	0.016	0.024
Eugenol	AOO	9	8	8	0	1	1	0	14.3	13.8
Eugenol	ACE	2	1	1	0	1	0	1	18.2	NC
Formaldehyde	DMF	2	1	1	0	1	1	0	0.27	0.11
Glutaraldehyde	DMF	2	1	1	0	1	1	0	0.07	0.09
HCA	ACE	5	4	4	0	1	1	0	5.8	12.9
Isoeugenol	AOO	33	32	32	0	1	1	0	1.4	0.8
Methyl salicylate	AOO	7	6	0	6	1	0	1	NC	NC
Nickel sulfate	DMSO	2	1	1	0	1	0	1	1.5	NC
Oxazolone	AOO	6	5	5	0	1	1	0	0.0018	IDR
Potassium dichromate	DMSO	10	8	8	0	2	1	1	0.09	0.2
Trimellitic anhydride	AOO	3	1	1	0	2	2	0	9.2	0.15
Total Studies		108	86	77	9	22	16	6		

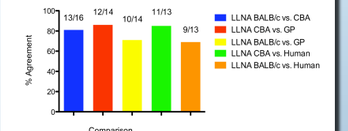
Abbreviations: Avg. = average; ACE = acetone; AOO = acetone-olive oil; DMF = dimethylformamide; DMSO = dimethyl sulfoxide; DNCB = dinitrochlorobenzene; DNFB = dinitrofluorobenzene; EC3 = estimated concentration needed to produce a stimulation index of three; HCA = α-hexylcinnamic aldehyde; IDR = insufficient dose-response, that is, result was positive but the dose-response was insufficient for the calculation of an EC3 (i.e., very low slope or nonmonotonic); LLNA = murine local lymph node assay; NC = not calculated because the result was negative; Neg = negative; No. = number; Pos = positive.

Table 3: Substances Discordant Between LLNA, GP and Humans

Chemical Name	LLNA Vehicle	Conc. (%)	SI	EC3 (%)	Mouse Strain	LLNA Call	LLNA Study Length (Days)	Overall LLNA Call[1] (CBA)	Overall LLNA Call[1] (BALB/c)	Overall GP[2] Call	Overall Human[3] Call	LLNA Reference	GP Reference	Human Reference
Eugenol	ACE	25, 50, 75	5.4, 10.6, 10.5	18.5	CBA/J	+	5	+	-	+	+	Gerberick et al. (1992)	Basketter et al. (1999)	Basketter et al. (1999)
		10, 20	1.07, 1.89	NC	BALB/c	-	4					Sailstad et al. (1995)		
Cobalt chloride	DMSO	0.5, 1.0, 2.5	3.2, 3.7, 2.8	0.4	CBA/Ca	+	5	+	-	+	+	Basketter and Scholes (1992)	Basketter et al. (1999)	Kligman (1966)
		0.5, 1.0, 2.5, 5.0	2.1, 3.5, 3.8, 7.2	0.8	CBA/N	+	4					Ikarashi (1992b)		
		1.0, 2.5, 5.0	1.5, 1.6, 2.7	NC	BALB/c	-	4					Mandervelt et al. (1997)		
Nickel sulfate	DMSO	0.25, 0.5, 1, 2.5, 5	1.3, 1.4, 1.4, 1.8, 3.1	4.8	CBA/J	+	6	+	-	+	+	Ryan et al. (2002)	Basketter and Scholes (1992)	Kligman (1966)
		2.5, 5	2.19, 2.46	NC	BALB/c	-	4					Ikarashi et al. (1992a)		

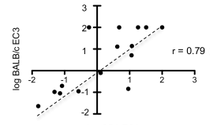
Abbreviations: ACE = acetone; Conc. = concentration; DMSO = dimethyl sulfoxide; EC3 = estimated concentration needed to produce a stimulation index of three; GP = guinea pig; LLNA = murine local lymph node assay; NC = not calculated since SI < 3.0; SI = stimulation index.
[1]An LLNA result was deemed positive if a stimulation index (SI) ≥ 3.0 occurred at any test concentration.
[2]*GP* refers to outcomes obtained by studies conducted using either the guinea pig maximization test or the Buehler test.
[3]*Human* refers to outcomes obtained by studies conducted using the human maximization test.
(-) = nonsensitizer, (+) = sensitizer

Figure 3: Comparison of LLNA Results Using CBA or BALB/c Mice



Abbreviations: GP = guinea pig skin sensitization outcomes; LLNA = murine local lymph node assay. Bar labels show the data on which the percentage calculation is based. Denominator is the number of substance-vehicle groups (eugenol was tested in two different vehicles, acetone and acetone-olive oil) GP refers to outcomes obtained by studies conducted using either the guinea pig maximization test or the Buehler test.
Human refers to outcomes obtained by studies conducted using the human maximization test or the inclusion of the test substance in a human patch test allergen kit.

Figure 4: Correlation of Results Obtained from LLNA Studies with CBA and BALB/c Mice



Log-transformed geometric mean EC3 values for 15 of the 16 substance-vehicle groups shown in Table 2. *r* = Spearman's Rank correlation coefficient.
NOTE: An EC3 value of 100% was assigned to negative LLNA results in order to exceed all positive values, so that they could be included in the correlation analysis.

- Oxazolone was not included in this analysis because the dose response obtained with BALB/c mice was inadequate to allow calculation of an EC3 value.
- Spearman's rank correlation is used for rating the extent of agreement with the "true" ranking of a set of observations (Steel and Torrie, 1980).
  - In this analysis, the CBA EC3 results were considered the "true" ranking.
- A highly significant ($p = 0.0005$) positive correlation ($r = 0.79$) was obtained between EC3 values calculated from LLNA studies in both strains (**Figure 4**).
  - Among the 10 substances for which an EC3 was calculated in both CBA and BALB/c studies, 5/10 were lower in CBA and 5/10 were lower in BALB/c (**Table 2**).

Discordant Results

- **Table 3** contains LLNA data for 3 substances for which the overall LLNA results were different between CBA and BALB/c mice, or between one mouse strain and guinea pig or human reference data.
  - In LLNA studies for cobalt chloride and nickel sulfate, the LLNA results using CBA mice were concordant with guinea pig and human reference tests, while those using BALB/c were discordant.
    - The discordant results obtained in BALB/c were based on a single study for each metal compound, and the maximum SI (2.7) was near the threshold for a positive response (3.0).
  - The negative study for nickel sulfate using BALB/c was a 4-day study, while the positive study in strain CBA was a 6-day study. Furthermore, the positive result in CBA mice was based on a maximum SI (3.1) that was near the threshold for a positive response (CBA maximum SI = 3.1; BALB/c maximum SI = 2.46; **Table 3**).
  - Therefore, there is insufficient information to draw definitive conclusions about the LLNA responses to metals when using either BALB/c or CBA mice.
- In the LLNA studies for eugenol with acetone as the vehicle, the LLNA results using CBA mice were concordant with guinea pig and human reference tests, while those using BALB/c mice were discordant.
  - The differences between the CBA and BALB/c studies may be due to the large differences in the concentration ranges used, where the maximum concentration used in the CBA study was almost 4-fold higher than that used in the BALB/c study.
  - It should also be noted that the BALB/c and CBA studies for eugenol in which AOO was used as the vehicle were both positive.

Conclusions

- Current testing guidelines (EPA, OECD) recommend using CBA mice unless it is sufficiently demonstrated that significant strain-specific differences in the LLNA response do not exist.
- When compared to LLNA studies using CBA mice (the strain specified in the ICCVAM-recommended LLNA protocol [ICCVAM 2009]), results of studies done on the same substances in BALB/c mice were in agreement most of the time (81% [13/16]) (**Figure 3**).
  - There was a positive correlation ($r = 0.79$) between EC3 values ($p = 0.0005$) (**Figure 4**).
  - Where there were different outcomes (n = 3) between the two mouse strains, the CBA studies were positive while the BALB/c studies were negative (**Table 3**).
  - These positive CBA study results were concordant with the human and GP outcome (**Table 3**).
- These results suggest that further characterization of strain and substrain differences is needed.
- Until such additional information becomes available caution should be used prior to selecting a strain other than CBA for use in the LLNA for regulatory testing.

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